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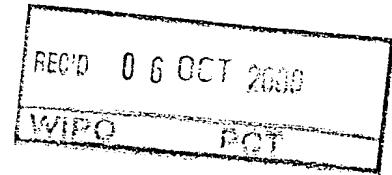
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E T U O I K E U S T O D I S T U S  
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# The gene cluster involved in aclacinomycin biosynthesis, and its use for genetic engineering

## Field of the invention

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This invention relates to the gene cluster for aclacinomycin biosynthesis derived from *Streptomyces galilaeus*, and the use of the genes included therein to obtain hybrid antibiotics, or to increase yields of aclacinomycins or related antibiotics.

## 10 Background of the invention

Anthracyclines are widely used anticancer agents. Seven different anthracyclines are in worldwide clinical use: daunorubicin, doxorubicin, idarubicin, epirubicin, pirarubicin, zorubicin and aclarubicin. A representative compound is doxorubicin, being the most 15 efficient and acting on a wide array of malignancies. A variety of toxic effects, like cumulative cardiotoxicity found with doxorubicin has sometimes led to discontinuation of the treatment. Furthermore, there are some type of malignancies which do not respond to available anthracyclines. The mechanism of action of anthracyclines, reflecting to their clinical efficiencies, is not clear, although most researchers consider 20 inhibition of topoisomerase II as a desired effect. Generation of free radicals derived from quinonic structures is suggested to be related to side effects such as cardiotoxicity. Anthracyclines have recently been reviewed by Professor Strohl and his group (1997).

Aclacinomycin A (aclarubicin) first described by Oki *et al.* (1975) is an anthracycline 25 antibiotic produced by *Streptomyces galilaeus* ATCC 31133 and *S. galilaeus* ATCC 31615. It is active against tumor cells and exhibits alleviated toxic properties as compared with doxorubicin. However, its activity does not reach solid tumors, limiting its use in leukemia treatment. Aclarubicin differs from the other counterparts in its structure. A trisaccharide moiety, rhodosamine-2-deoxyfucose-cinerulose A is attached at C-7 by a 30 glycosidic bond, whereas at the corresponding position of daunomycins only one sugar residue, daunosamine, is attached.

Despite the long history of anthracyclines, three decades or so, the studies on their biosynthesis are still going on, and there is further interest to obtain novel molecules for

the development of cancer chemotherapeutics. A method currently used for finding novel molecules for drug screening is genetic engineering. Cloning the genes for anthracycline biosynthesis facilitates the production of hybrid anthracyclines, as well as their use in combinatorial biosynthesis to generate novel molecules. As regards the 5 chemical nature of anthracyclines currently in clinical use, aclarubicin has unique features which make its biosynthetic genes interesting in creating novel products.

Regarding the genes for deoxyhexose pathway, Madduri *et al.* (1998) have reported that a gene derived from avermectin biosynthesis cluster caused the production of hybrid 10 anthracyclines altering a sugar moiety when transferred into a *S. peucetius* strain. The product obtained was epirubicin, a commercially important anthracycline. In this case a hydroxy group in the daunosamine moiety was in the opposite stereochemistry due to the action of an avermectin biosynthesis gene.

15 *S. galilaeus* has been used as the host to prepare hybrid anthracyclines using the genes derived from rhodomycin pathway from *S. purpurascens* (Niemi *et al.*, 1994) and from nogalamycin biosynthesis cluster from *S. nogalater* (Ylihonko *et al.*, 1996a). The genes for nogalamycin pathway were used to generate the hybrid anthracycline production in *S. steffisburgensis* producing typically steffimycin (Kunnari *et al.*, 1997). Previously, 20 biosynthesis genes for actinorhodin have been expressed in *S. galilaeus*, resulting in the formation of aloesaponarin (Strohl *et al.*, 1991). These hybrid compounds were modified in the aglycone moiety. Recently, the biosynthesis genes involved in deoxyhexose pathway of nogalamycin were used to generate hybrid compounds using the *S. galilaeus* mutants as hosts (FI pat. appln No. 982295).

25

As shown above, *S. galilaeus* has been used as a cloning host to generate novel molecules, whereas its use to donate the genes has not been described. The identified genes involved in aclacinomycin biosynthesis include polyketide reductase gene (Tsukamoto *et al.*, 1994), aklanonic acid methyl ester cyclase (GeneBank, ACCESSION 30 AF043550) and genes for polyketide synthase (Hutchinson and Fujii, 1995; the sequence not available).

### Summary of the invention

The present invention concerns a gene cluster, most of the genes of which are derived from deoxyhexose pathway for rhodosamine, 2-deoxyfucose and/or rhodinose. The gene 5 cluster was cloned from *S. galilaeus* ATCC 31615 and it is involved in biosynthesis of aclacinomycins.

### Detailed description of the invention

10 The experimental procedures of the present invention include biochemical and chemical methods conventional in the art. Detailed description of the techniques not explained here are given in the manuals by Hopwood *et al.* 'Genetic manipulation of Streptomyces: a laboratory manual'. The John Innes Foundation, Norwich (1985) and by Sambrook *et al.* (1989) 'Molecular cloning: a laboratory manual'.

15 The publications, patents and patent applications cited herein are given in the reference list in their entirety.

20 The present invention concerns particularly the discovery of the gene cluster for aclacinomycin biosynthesis. The cluster, when introduced into *S. peucetius* strains caused the production of hybrid antibiotics modified in their sugar moiety.

25 Several strategies may be adopted to clone genes for an antibiotic. Using *E. coli* as a host for a gene library, hybridization is the most advantageous screening strategy. The probe for hybridization may be any known fragment that shows sufficient homology to the biosynthetic cluster for aclarubicin sugars, to be able to hybridize with said cluster. A DNA fragment which is identical to the desired region is preferred. Such a fragment, called Sg-dht, was obtained by PCR amplification of *S. galilaeus* chromosomal DNA, using degenerated oligonucleotides annealing to the conserved region of 4,6-dehydratase 30 gene. 4,6-dehydratase is the first enzyme participating to a reaction series that converts a glucose molecule bound to a nucleotide into 6-deoxy sugars generally found in antibiotics. Using this probe it was possible to clone the cluster of deoxyhexose pathway

from a restricted gene library. To simplify the cloning strategy the library was prepared in a pUC-based plasmid (e.g. pBluescript or pWHM1109) replicating in *E. coli*.

The strategy to clone the genes involved in aclacinomycin biosynthesis according to the invention was in brief: Total DNA was isolated from *S. galilaeus* (ATCC 31615) and digested with several restriction enzymes that yield fragments of 10 kb in average. Restriction fragments were analyzed by Southern hybridization using a homologous DNA fragment, Sg-dht, as a probe. *Bgl*II gave a hybridized fragment of 8.5 kb, and a double digestion with *Xho*I and *Not*I gave a hybridized fragment of 7 kb. DNA digestion using (i) *Bgl*II and (ii) *Xho*I-*Not*I was carried out and the fragments were ligated to the *E. coli*-*Streptomyces* shuttle vector, pWHM1109, digested with *Bam*HI and to the pBluescript digested with *Xho*I-*Not*I, respectively. The ligation mixtures were introduced into *E. coli* XL1BlueMRF' that exhibits alleviated restriction-modification systems. Colonies were plated on the agar plates in the dilution to give 200 to 600 cfu (colony forming units) per plate. Well grown colonies were transferred in nylon membranes for hybridization, which was carried out using the Sg-dht probe. Six out of the 786 *Bgl*II-digested clones gave hybridization signal and 7 out of 1523 of those clones carrying *Xho*I-*Not*I fragments. Hybridization and washes were carried out in the stringent conditions of 65°C in a low salt concentration. Several techniques for the labeling of the probe and for hybridization are possible, but the procedure according to Boehringer Mannheim's "The DIG System User's Guide for Filter Hybridization" is preferred. The colonies giving hybridization signals were cultivated for plasmid isolation. The plasmids were analyzed by Southern hybridization to confirm the reliability of the colony hybridization. Plasmids containing the desired DNA fragments (Sg4 and Sg5) were designated as pSgc4 (*Bgl*II-fragment) and pSgc5 (*Xho*I-*Not*I fragment)(see Fig. 2).

The fragments, Sg4 and Sg5, were subcloned for sequencing in *E. coli* vectors pUC19 and pBluescript. In total 30 subclones were used to obtain the nucleotide sequence of Sg4 and Sg5. The sequenced cluster revealed thirteen genes involved in biosynthesis of aclacinomycins. Comparison with the sequences found in the sequence library suggested the functions as *sga2* for an activator, *sga3* for a dehydratase, *sga4* for oxidoreductase, *sga5* for dTDP-glucose 4,6-dehydratase, *sga6* for glycosyl transferase (GTF), *sga7* for

a putative isomerase, *sga8* for aklaviketone reductase, *sga9* for a putative polyketide assembler, *sga10* for a putative cyclase, *sga11* for aminomethylase, *sga12* for glucose-1-phosphate thymidylyl transferase, *sga13* for aminotransferase. The function of *sga1* is not suggested based on similarity searches. Based on the deduced functions, nine genes 5 are involved in glycosylation pathway. The genes involved in the formation of aglycone are *sga8*, *sga9*, and *sga10*. The activator, Sga2, may control both the glycosylation system and the formation of aklavinone via polyketide pathway.

Sg4 derived from pSgc4 was cloned in the *Streptomyces* expression vector pIJE486 10 (Ylihonko *et al.*, 1996b) in *S. lividans* TK24 to give pSgs4. This vector is a high copy number plasmid that replicates in several *Streptomyces* spp. (Ward *et al.*, 1986) and it contains a constitutively expressed promoter, *ermE* (Bibb *et al.*, 1985) upstream from the multiple cloning site. The plasmid pSgs4 isolated from TK24 was introduced into the *S. galilaeus* strains that are blocked in deoxyhexose pathway of aclacinomycin 15 biosynthesis and into the *S. peucetius* mutants producing  $\epsilon$ -rhodomycinone based on a lesion in glycosylation genes. The ability of aclacinomycin production was restored by three *S. galilaeus* mutants, H063, H054 and H065. The mutant strain H063 accumulates aklavinone and it was completely complemented by the plasmid pSgs4. Instead, H054 and H065 producing aklavinone glycosides sharing neutral sugars, but not rhodosamine, 20 were only partially complemented by pSgs4. Surprisingly, H063 carrying pSgs4 (H063/pSgs4) was able to produce aclacinomycins two-fold to that of the wild type *S. galilaeus*. *S. peucetius* M18 and M90 which produce  $\epsilon$ -rhodomycinone were selected to hosts for pSgs4. L-rhamnosyl- $\epsilon$ -rhodomycinone (El Khamed *et al.*, 1977) was obtained 25 when pSgs4 was expressed in the mutants M18 and M90 and, in addition, M18/pSgs4 produced L-daunosaminyl- $\epsilon$ -rhodomycinone (Essery and Doyle, 1980). The structures were not new ones but this demonstrates the ability of the gene cluster according to the present invention to generate hybrid products in a heterologous host. To produce hybrid compounds we prefer to use E1 medium supplemented with a suitable antibiotic, in this case, thiostrepton, to maintain the selection pressure for the plasmid containing strains. 30 The products were extracted by organic solvents and purified by chromatography to obtain the compounds in high purity for structural elucidation.

Examples to further illustrate the invention are given hereafter.

### Brief description of the drawings

**FIG. 1** shows the structures of aclacinomycin, daunomycin and  $\epsilon$ -rhodomycinone.

5 **FIG. 2** is a diagram of the gene cluster for aclacinomycin biosynthesis.

**FIG. 3** describes the proposed biosynthesis pathway for sugars found in aclacinomycins.

10 **FIG. 4** shows the structures of the hybrid compounds produced by M18/pSgs4 (1 and 2) and M90/pSgs4 (2).

## EXPERIMENTAL

### 15 Materials used

Restriction enzymes used were purchased from Promega (Madison, Wisconsin, USA), Fermentas (Lithuania) or Boehringer Mannheim (Germany), alkaline phosphatase from Boehringer Mannheim, and used according to manufacturers' instructions. Proteinase K 20 was purchased from Promega and lysozyme from Sigma. Hybond<sup>TM</sup>-N nylon membranes used in hybridization were purchased from Amersham (Buckinghamshire, England), DIG DNA Labelling Kit and DIG Luminescent Detection Kit from Boehringer Mannheim. Qiaquick Gel Extraction Kit from Qiagen (Hilden, Germany) was used for isolating DNA from agarose.

25

### Bacterial strains and their use

*Escherichia coli* XL1BlueMRF' (Stratagene, La Jolla, California) was used for cloning.

30 *Streptomyces lividans* TK24 was the first cloning host for gene expression. The strain was provided by prof. Sir David Hopwood, John Innes Centre, UK.

The wild type, *Streptomyces galilaeus* ATCC 31615, produces aclacinomycins. It was used here to donate the genes of the invention.

5 *Streptomyces galilaeus* H039 (Ylihonko *et al.*, 1994) produces Akv-(Rho)<sub>0-3</sub>. It was used as an expression host for pSgs4 being more easily transformed than the other mutants or the wild type.

10 *Streptomyces galilaeus* H054 (Ylihonko *et al.*, 1994) produces Akv-Rho-dF-(CinA)<sub>0-1</sub>, Akv-dF-dF-(CinA)<sub>0-1</sub> and Akv-dF-Rho-Rho. It was used as an expression host for pSgs4.

*Streptomyces galilaeus* H063 produces aklavinone. It is a mutant strain derived from the wild type *S. galilaeus*. H063 was used as an expression host for pSgs4.

15 *Streptomyces galilaeus* H065 produces aklavinone with neutral glycosides. It is a mutant strain derived from the wild type *S. galilaeus*. H065 was used as an expression host for pSgs4.

20 *Streptomyces peucetius* M18 and M90 producing  $\epsilon$ -rhodomycinone are the mutants derived from *S. peucetius* var. *caesius* (ATCC 27952). They were used as expression hosts for pSgs4.

### Plasmids

25 *E. coli* cloning vectors pBluescript SK (Stratagene) and pUC19 (Pharmacia, Sweden) were used for making the subclones for sequencing and pBluescript was used also as a vector of a gene library.

30 pWHD1109 (provided by prof CR Hutchinson, Wisconsin, USA) is a shuttle vector replicating in *E. coli* and in streptomycetes. It was used as a vector of a gene library.

pIJ486 is a high copy plasmid vector provided by prof. Sir David Hopwood, John Innes Centre, UK (Ward *et al.*, 1986).

pJJE486 (Ylihonko *et al.*, 1996b) is an expression vector containing *ermE* (Bibb *et al.*, 1985) to promote expression of the cloned genes.

### Nutrient media and solutions

5

For cultivation of *S. galilaeus* for total DNA isolation TSB medium was used. Lysozyme solution (0.3 M sucrose, 25 mM Tris, pH 8 and 25mM EDTA, pH 8) was used to isolate total DNA. TE buffer (10 mM Tris, pH 8.0 and 1mM EDTA) was used to dissolve DNA.

10

### TRYPTONE-SOYA BROTH (TSB)

Per litre: Oxoid Tryptone Soya Broth powder 30 g.

### ISP4

15 Bacto ISP-medium 4, Difco; 37 g/l.

### E1 Per litre in tap water:

	glucose	20 g
	soluble starch	20 g
20	Farmamedia	5 g
	Yeast extract	2.5 g
	K <sub>2</sub> HPO <sub>4</sub> •3H <sub>2</sub> O	1.3 g
	MgSO <sub>4</sub> •7H <sub>2</sub> O	1 g
	NaCl	3 g
25	CaCO <sub>3</sub>	3 g

pH adjusted to 7.4 before autoclaving

### General methods:

30 NMR data was collected with a JEOL JNM-GX 400 spectrometer. <sup>1</sup>H and <sup>13</sup>C NMR samples were internally referenced to TMS.

The anthracycline metabolites were determined by (i) HPLC (LaChrom, Merck Hitachi, pump L-7100, detector L-7400 and integrator D-7500) using a LiChroCART RP-18

35 column. Acetonitrile:potassium hydrogen phosphate buffer (60 mM, pH 3.0 adjusted

with citric acid) was used as a mobile phase. Gradient system starting from 65 % to 30 % of potassium dihydrogen phosphate buffer was used to separate the compounds. The flow rate was 1 ml/min and the detection was carried out at 480 nm, and (ii) by TLC using precoated Kieselgel 60 F<sub>254</sub> glass plates (Merck, Darmstadt, Germany) with an 5 elution solution of toluene:ethyl acetate:methanol:formic acid (50:50:15:3).

ISP4 plates supplemented with thiostrepton (50 µg/ml) were used to maintain the plasmid carrying cultures.

## 10 Example 1. Cloning the gene cluster for aclacinomycin biosynthesis

### 1.1 Selection of clones by hybridization

For isolation of total DNA, *Streptomyces galilaeus* was grown for four days in 50 ml of 15 TSB medium supplemented with 0.5% glycine. The cells were harvested by centrifuging for 15 min (3900 x g) in 12 ml Falcon tubes, and stored at -20°C. Cells from a 50 ml culture were used to isolate DNA. 5 ml of lysozyme solution containing 5 mg/ml of lysozyme was added on the cells of each Falcon tube, and incubated for 20 min at 37°C. 500 µl of 10% SDS containing 0.7 mg of proteinase K was added on the cells, 20 and incubated for 80 min at 62°C, another 500 µl of 10% SDS containing 0.7 mg of proteinase K was added, and incubation was continued for 60 min. The sample was chilled on ice and 600 µl of 3M NaAc, pH 5.8 was added, and the mixture was extracted with equilibrated phenol (Sigma). The phases were separated by centrifuging (1400 x g) for 10 min. The DNA was precipitated from the water phase with an equal 25 volume of isopropanol and collected by spooling with a glass rod and washed by dipping into 70% ethanol, air dried and dissolved in 500 µl of TE-buffer.

Southern hybridization to determine suitable restriction enzymes for preparing the 30 restricted plasmid libraries was carried out using *Bgl*II, *Xho*I, *Not*I and their combinations. A fragment of about 9 kb hybridizing with the Sg-dht probe was preferred. For hybridization 600 ng of digested *S. galilaeus* DNA was loaded onto the agarose gel and after electrophoresis, the DNA was transferred from the gel to a nylon membrane by vacuum blotting. Hybridization was carried out according to Boehringer Mannheim's

manual 'The DIG System User's Guide for Filter Hybridization'. The probe for hybridization, Sg-dht, which was used for colony hybridization as well, was obtained by amplifying a gene fragment from the *S. galilaeus* DNA which is internal to the 4,6-dehydratase gene and corresponds to the fragment of 6345 to 6861 shown in SEQ ID NO:14. PCR was used for amplification, and the sequences for the degenerated oligonucleotide primers were 5'-CSGGSGSSGCSGGSTTCATSGG-3' (forward, SEQ. ID. NO:15) and 5'-GGGWRCTGGYRSGGSCCGTAGTTG-3' (reverse, SEQ. ID. NO:16). Suitable fragments were a 9 kb *Bgl*II fragment and a 7 kb *Xho*I-*Not*I fragment.

10 Ten micrograms of the chromosomal DNA was digested with *Bgl*II. The DNA fragments were separated by agarose gel electrophoresis and the band of 8 to 9 kb were cut from the 0.6% low gelling temperature SeaPlaque® agarose. The DNA band was isolated from the gel using Qiagen Gel Extraction Kit. The isolated fragment was ligated to pWHM1109 plasmid vector digested with *Bam*HI and defosforylated, in the ratio of 3 moles of the insert DNA to 1 mole of the vector DNA. The ligated DNA was introduced into *E. coli* XL1BlueMRF' by electroporation. Using the whole ligation mixture 786 colonies were obtained. The colonies were grown on agar plates for at least 12 h and transferred to nylon membranes. Hybridization of colony membranes was carried out as Southern using Sg-dht as a probe. Six clones gave signal in hybridization and the corresponding colonies were plated on agar and inoculated in 3 ml of LB medium for isolation of the plasmid DNA. Southern hybridization was used to study whether the plasmids derived from the clones carried the desired insert. Four of these plasmids contained the 4,6-dehydratase gene fragment and gave the identical restriction map thus carrying the same fragment representing both orientations. The fragment was designated as Sg4 and the plasmid containing the fragment as pSgc4.

In the same manner the plasmid library representing a 7 kb *Xho*I-*Not*I DNA fragment derived from *S. galilaeus* was constructed. pBluescript was digested with *Xho*I-*Not*I and the library containing the gene fragments of around 7 kb was constructed. In total 1523 colonies were hybridized and seven turned to be the desired clone. As described above, the clones were studied for the *Xho*I-*Not*I fragment. The insert fragment was designated as Sg5 and the plasmid as pSgc5. The strain *E. coli* XL1Blue MRF'/pSgc5 obtained was deposited according to the rules of the Budapest Treaty at Deutsche Sammlung von

Mikroorganismen und Zellkulturen GmbH (DSMZ) on August 12, 1999 with the accession number DSM 12999. The fragments Sg4 and Sg5 overlap within 836 bp corresponding bases from 6181 to 7016 in SEQ ID NO:14.

### 5 1.2. Subcloning the fragments for sequencing

To determine the nucleotide sequence of the whole cluster of the Sg4 and Sg5 suitable subclones were constructed. The convenient restriction sites were used for subcloning the 14806 bp region in the plasmids pUC19 and pBluescript. Nineteen subclones were 10 needed to sequence Sg4, and 11 subclones for Sg5.

*E. coli* XL1BlueMRF' cells containing the subcloned plasmids were cultivated overnight at 37°C in 5 ml of LB-medium supplemented with 50 µg/ml of ampicillin. To isolate plasmids for sequencing reactions Wizard Plus Minipreps DNA Purification 15 System kit of Promega or Biometra Silica Spin Disc Plasmid DNA Miniprep kit of Biomedizinische Analytik GmbH were used according to the manufacturers' instructions.

DNA sequencing was performed using the automatic ABI DNA sequencer (Perkin-Elmer) according to the manufacturer's instructions.

20

### 1.3 Sequence analysis and the deduced functions of the genes

Sequence analyses were made using the GCG sequence analysis software package (Version 8; Genetics Computer Group, Madison, Wis., USA). The translation table was 25 modified to accept also GTG as a start codon. Codon usage was analyzed using published data (Wright and Bibb 1992).

According to the CODONPREFERENCE program the sequenced DNA fragment revealed 11 complete open reading frames (ORFs), and two 5' ends of the other ORFs 30 (sga1 and sga13). The functions of the genes were concluded by comparing the amino acid sequences translated from their base sequences to the known sequences in the data banks. The results are shown in Table 1 referring to the sequence data given in the application.

The suggested functions for the genes match well with a proposed biosynthetic pathway of sugars of aclacinomycins (Fig. 3). The last residue in a trisaccharide moiety of aclacinomycins is rhodinose that is enzymatically converted to cinerulose. Aclacinomycin N, a precursor of aclarubicin, contains rhodinose as the third sugar residue.

5

**Table 1.**

Gene	Position	Amino acids	Deduced function	Remarks
<i>sga1</i>	-1986 compl	>662	unknown	not complete Seq.ID.NO:1
<i>sga2</i>	2523-3341	272	activator	Seq.ID.NO:2
<i>sga3</i>	3355-4659 compl	434	dehydratase	Seq.ID.NO:3
<i>sga4</i>	4821-5810	329	oxidoreductase	Seq.ID.NO:4
<i>sga5</i>	5920-6891 compl	323	dTDP-glucose 4,6-dehydratase	Seq.ID.NO:5
<i>sga6</i>	6879-8210 compl	443	glycosyl transferase (GTF)	Seq.ID.NO:6
<i>sga7</i>	8287-9618 compl	443	putative isomerase	Seq.ID.NO:7
<i>sga8</i>	9642-10445 compl	267	aklaviketone reductase (KRII)	Seq.ID.NO:8
<i>sga9</i>	10471-10905 compl	144	putative polyketide assembler	Seq.ID.NO:9
<i>sga10</i>	11115-11894	259	putative cyclase	Seq.ID.NO:10
<i>sga11</i>	11956-12672	238	aminomethylase	Seq.ID.NO:11
<i>sga12</i>	12685-13560 compl	291	glucose-1-phosphate thymidyltransferase	Seq.ID.NO:12
<i>sga13</i>	13783-14805	341	aminotransferase	Seq.ID.NO:13 not complete

#### 1.4 Expression cloning in *Streptomyces* strains

25

The 8 kb *Bam*HI-*Hind*III fragment from pSgc4 was ligated in pJJE486 to give pSgs4. Plasmid pSgs4 was introduced into *S. lividans* TK24 by protoplast transformation. The strain *S. lividans* TK24/pSgs4 obtained was deposited according to the rules of the

Budapest Treaty at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) on August 12, 1999 with the accession number DSM 12998. The plasmid pSgs4 was isolated from the strain, and further transferred into *S. galilaeus* mutant H039. The plasmid preparate isolated from H039 was subsequently introduced into 5 H063, H054, and H065 mutants deficient of glycosylation system of aclacinomycins. The usage of H039 as a primary *S. galilaeus* host was due to the better efficiency for the intake of foreign DNA.

*S. galilaeus* mutants were studied for complementation by cultivating the clones 10 containing pSgs4 in E1 medium supplemented with thiostrepton (10  $\mu$ g/ml). The products from a 500  $\mu$ l sample of the culture broth were extracted with toluene:methanol (1:1) at pH 7. The metabolites from both the transformed clones and the mutants were analyzed by TLC and HPLC to find the differences caused by pSgs4. H063 producing endogenously aklavinone was restored to aclacinomycin producer with 15 pSgs4. No aklavinone was found in the culture broth of H063/pSgs4. However, complementation was not completed when pSgs4 was expressed in H054 and H065. Both of the mutants produce aklavinone with neutral glycosides. Incomplete complementation was presumably due to the loss of the plasmids of some bacterial cells during cultivation, or a low expression of the genes needed as an activator is not present in 20 pSgs4.

In the same manner, pSgs4 isolated from TK24 was introduced into the *S. peucetius* mutants M18 and M90. The characteristic product for these mutants is  $\epsilon$ -rhodomycinone. The strains M18/pSgs4 and M90/pSgs4 containing the plasmid were cultivated in E1 25 medium supplemented with thiostrepton (10  $\mu$ g/ml), and the metabolites therein were analyzed by TLC and HPLC. Both of the clones revealed an altered production profile as compared with the products obtained from the mutants. M90/pSgs4 accumulated a glycosylated product, yielding  $\epsilon$ -rhodomycinone as the aglycone. The compound was identified as L-rhamnosyl- $\epsilon$ -rhodomycinone which has been previously synthesized 30 (CAS=63252-11-9) by El Khamed *et al.* (1977).

M18/pSgs4 produced two compounds differing from the parental strain. According to the HPLC and TLC data one compound was the same as was produced by M90/pSgs4,

L-rhamnosyl- $\epsilon$ -rhodomycinone, and the other one was L-daunosaminyl- $\epsilon$ -rhodomycinone, which was previously characterized by Essery and Doyle (1980).

**Table 2:** TLC and HPLC data of the hybrid products

5

Product	Rf-value	Retention time
$\epsilon$ -rhodomycinone	0.67	6.70
L-rhamnosyl- $\epsilon$ -rhodomycinone	0.38	5.00
L-daunosaminyl- $\epsilon$ -rhodomycinone	0.04	4.06

10

### 1.5 Applicability of pSgs4 for strain improvement

Since H063 was completely complemented by pSgs4, the production level of aminoglycosides was studied. For this purpose, H063/pSgs4, H063 and the wild type *S. galilaeus* were cultivated in E1 medium in the Erlenmeyer bottles for four days. Two samples of 2 ml from each culture were extracted first with toluene:methanol (1:1) in acidic conditions to remove the neutral glycosides and the aglycones. The extraction procedure was repeated until neutral glycosides and the aglycones had disappeared from the water phase. The amount of anthracycline metabolites in toluene phase was determined and is shown in Table 3. Aclacinomycins containing rhodosamine were extracted from the water phase by chloroform. Both toluene and chloroform extracts were analyzed by TLC and toluene phases contained mostly aklavinone and the degradative products. Chloroform phases contained mainly aminoglycosides, although minor amounts of the aglycones were also detected. Extracts were evaporated to dryness and subsequently dissolved into 1 ml of methanol. The amounts of anthracycline metabolites were detected by spectrophotometer at 430 nm. The amounts related to absorbance were calculated using an extinction coefficient of 13000. The results given as mg/l of cultivation broth are shown in Table 3. The production of aclacinomycins by H063/pSgs4 was at least twofold better than obtained by the wild type.

**Table 3.**

Sample	Chloroform phase aminoglycoside fraction		Toluene phase aglycone fraction	
	Absorbance	Concentration (mg/l)	Absorbance	Concentration (mg/l)
H063	0.401	12.6	2.956	92.3
H063/pSgs4	2.751	85.9	2.974	92.9
<i>S. galilaeus</i>	1.338	41.8	0.690	21.5

5 10 The ability to increase the yield of aclacinomycins by pSgs4 in the mutant H063 suggests that the genes according to the present invention are useful in strain improvement.

15 **Example 2. Compounds generated by pSgs4**

10 The seed culture, 180 ml of E1 culture of the plasmid containing strains, M18/pSgs4 or M90/pSgs4, was obtained by cultivating each of the strains in three 250 ml Erlenmeyer flasks containing 50 ml of E1-medium supplemented with thiostrepton (5 µg/ml) for four days at 30°C, 330 rpm. The combined culture broths (180 ml) were used to

20 inoculate 13 l of E1-medium in a fermentor (Biostat E). Fermentation was carried out for five days at 28°C (330 rpm, aeration: 450 l/min).

25 The cells were harvested by centrifuging. 2.6 l of methanol was used to brake the bacterial cells. The anthracycline metabolites were extracted from methanol solution at pH 8 using 2 l of ethyl acetate and the extract was evaporated to dryness. The viscous residue was loaded onto a silica column of 4 × 10 cm and toluene:ethyl acetate:formic acid (50:50:3) with increasing amount of methanol was used as an eluent. Pure fractions were pooled and extracted with 1M phosphate buffer (pH 8.0) and water. Organic phase

was dried with anhydrous  $\text{Na}_2\text{SO}_4$  and then treated with hexane to effect precipitation. Pure compounds appeared as red powders dried under vacuum.

Complete structural determination of the compounds were accomplished by NMR.

5 Proton and carbon assignments were based on a conventional NOE difference, pHSQC and HMBC measurements. Connectivities in particular relied heavily on HMBC experiment.

As deduced from the data given in Table 4, the structures revealed were L-rhamnosyl-  
10  $\epsilon$ -rhodomycinone (1) and L-daunosaminyl- $\epsilon$ -rhodomycinone (2) shown in Figure 4.

Although these structures were not novel, the generation of the hybrid products by the genes involved in glycosylation portion of aclacinomycin biosynthesis well demonstrates that the genes of pSgs4 are functional and ready to use in drug discovery for finding  
15 novel molecules.

#### **Deposited microorganisms**

The following microorganisms were deposited according to the Budapest Treaty at  
20 Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Masche-  
roder Weg 1b, D-38124 Braunschweig, Germany.

	<b>Microorganism</b>	<b>Accession number</b>	<b>Date of deposit</b>
25	<i>S. lividans</i> TK24/pSgs4	DSM 12998	12 August 1999
	<i>E. coli</i> XL1BlueMRF'/pSgc5	DSM 12999	12 August 1999

**Table 4.**  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts of **1** ( $\text{DMSO}_{\text{d}6}$ ) and **2** (trace of TFA in  $\text{DMSO}_{\text{d}6}$ ) in 400 and 100 MHz respectively.

Site	<b>1</b> $^1\text{H}$	<b>1</b> $^{13}\text{C}$	<b>2</b> $^1\text{H}$	<b>2</b> $^{13}\text{C}$
1	7.74, 1H, dd, 7.5, 0.9	118.9(d)	7.74, 1H, dd, 7.5, 1.0	119.7(d)
2	7.64, 1H, dd, 8.4, 7.5	136.5(d)	7.68, 1H, dd, 8.1, 7.5	137.4(d)
3	7.22, 1H, dd, 8.4, 0.9	124.1(d)	7.24, 1H, dd, 8.1, 1.0	125.0(d)
4	—	161.8(s)	—	162.6(s)
4-OH	12.00, 1H, s	—	exchange broadened	—
4a	—	115.2(s)	—	115.9(s)
5	—	189.9(s)	—	190.6(s)
5a	—	110.4(s)	—	111.4(s)
6	—	156.2(s)	—	157.1(s)
6-OH	13.41, 1H, s	—	exchange broadened	—
6a	—	135.1(s)	—	135.7(s)
7	5.14, 1H, d, 4.5	70.9(d)	5.15, 1H, d, 3.6	71.3(d)
8A	2.31, 1H, d, 15.1	28.9(t)	2.33, 1H, d, 14.6	34.0(t)
8B	2.14, 1H, dd, 15.1, 4.5	—	2.21, 1H, dd, 14.6, 3.8	—
9	—	70.0(s)	—	70.9(s)
10	4.16, 1H, s	51.2(d)	4.23, 1H, s	51.8(d)
10a	—	134.8(s)	—	136.1(s)
11	—	156.0(s)	—	156.8(s)
11-OH	12.77, 1H, s	—	exchange broadened	—
11a	—	110.8(s)	—	111.1(s)
12	—	185.4(s)	—	186.0(s)
12a	—	132.6(s)	—	133.3(s)
13A	1.73, 1H, dq, 13.9, 7.4	31.7(t)	1.83, 1H, dq, 14.1, 7.3	32.0(t)
13B	1.38, 1H, dq, 13.9, 7.4	—	1.47, 1H, dq, 14.1, 7.3	—
14	1.05, 3H, t, 7.4	6.09(q)	1.13, 3H, t, 7.3	6.90(q)
15	—	170.4(s)	—	171.1(s)
16	3.63, 3H, s	51.7(q)	3.70, 3H, s	52.3(q)
1'	5.28, 1H, brs	103.7(d)	5.52, 1H, d, 3.1	100.7(d)
2'	3.83, 1H, d, 5.2	70.9(d)	2.18, 2H, m	27.1(t)
3'	3.44, 1H, dd, 9.0, 5.2	70.8(d)	3.40, 1H, dd, 11.8, 5.1	55.5(d)
4'	3.41, 1H, dd, 9.1, 9.0	72.0(d)	3.98, 1H, brs	67.0(d)
5'	3.77, 1H, dq, 9.1, 6.2	68.9(d)	4.21, 1H, q, 6.3	65.3(d)
6'	1.29, 3H, d, 6.2	16.9(q)	1.32, 3H, t, 6.3	16.7(q)

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## SEQUENCE LISTING

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 20 25 30  
 Val Ala Ser Gln Pro Ala Leu Thr Ala Ser Ile Thr Ala Ala Gly Leu  
 35 40 45  
 Thr Ala Val Pro Val Gly Ala Asp Pro Arg Leu Asp Glu Met Val Lys  
 50 55 60  
 Gly Val Gly Asp Ala Val Leu Ser His His Ala Asp Gln Ser Leu Asp  
 65 70 75 80  
 Ala Asp Thr Pro Gly Gln Leu Thr Pro Ala Phe Leu Gln Gly Trp Asp  
 85 90 95  
 Thr Met Met Thr Ala Thr Phe Tyr Thr Leu Ile Asn Asp Asp Pro Met  
 100 105 110

Val Asp Asp Leu Val Ala Phe Ala Arg Gly Trp Glu Pro Asp Leu Ile  
 115 120 125

Leu Trp Glu Pro Phe Thr Phe Ala Gly Ala Val Ala Ala Lys Val Thr  
 130 135 140

Gly Ala Ala His Ala Arg Leu Leu Ser Phe Pro Asp Leu Phe Met Ser  
 145 150 155 160

Met Arg Arg Ala Tyr Leu Ala Gln Leu Gly Ala Ala Pro Ala Gly Pro  
 165 170 175

Ala Gly Gly Asn Gly Thr Thr His Pro Asp Asp Ser Leu Gly Gln Trp  
 180 185 190

Leu Glu Trp Thr Leu Gly Arg Tyr Gly Val Pro Phe Asp Glu Glu Ala  
 195 200 205

Val Thr Gly Gln Trp Ser Val Asp Gln Val Pro Arg Ser Phe Arg Pro  
 210 215 220

Pro Ser Asp Arg Pro Val Val Gly Met Arg Tyr Val Pro Tyr Asn Gly  
 225 230 235 240

Pro Gly Pro Ala Val Val Pro Asp Trp Leu Arg Val Pro Pro Thr Arg  
 245 250 255

Pro Arg Val Cys Val Thr Leu Gly Met Thr Ala Arg Thr Ser Glu Phe  
 260 265 270

Pro Asn Ala Val Pro Val Asp Leu Val Leu Lys Ala Val Glu Gly Leu  
 275 280 285

Asp Ile Glu Val Val Ala Thr Leu Asp Ala Glu Glu Arg Ala Leu Leu  
 290 295 300

Thr His Val Pro Asp Asn Val Arg Leu Val Asp His Val Pro Leu His  
 305 310 315 320

Ala Leu Leu Pro Thr Cys Ala Ala Ile Val His His Gly Gly Ala Gly  
 325 330 335

Thr Trp Ser Thr Ala Leu Val Glu Gly Val Pro Gln Ile Ala Met Gly  
 340 345 350

Trp Ile Trp Asp Ala Ile Asp Arg Ala Gln Arg Gln Gln Ala Leu Gly  
 355 360 365

Ala Gly Leu His Leu Pro Ser His Glu Val Thr Val Glu Gly Leu Arg  
 370 375 380

Gly Arg Leu Val Arg Leu Leu Asp Glu Pro Ser Phe Thr Ala Ala Ala  
 385 390 395 400

Ala Arg Leu Arg Ala Glu Ala Glu Ser Glu Pro Thr Pro Ala Gln Val  
 405 410 415

Val Pro Val Leu Glu Arg Leu Thr Ala Gln His Arg Ala Arg Glu Pro  
 420 425 430

Arg Arg Pro Gly Gly Thr Ser Pro Cys Val Ser  
 435 440

<210> 7  
 <211> 443  
 <212> PRT  
 <213> *Streptomyces galilaeus*

<400> 7  
 Val Gln Thr Gln Asn Ala Pro Glu Thr Ala Glu Asn Gln Gln Thr Asp  
 1 5 10 15  
 Ser Glu Leu Gly Arg His Leu Leu Thr Ala Arg Gly Phe His Trp Ile  
 20 25 30  
 Tyr Gly Thr Ser Gly Asp Pro Tyr Ala Leu Thr Leu Arg Ala Glu Ser  
 35 40 45  
 Asp Asp Pro Ala Leu Leu Thr Arg Arg Ile Arg Glu Ala Gly Thr Pro  
 50 55 60  
 Leu Trp Gln Ser Thr Thr Gly Ala Trp Val Thr Gly Arg His Gly Val  
 65 70 75 80  
 Ala Ala Glu Ala Leu Ala Asp Pro Arg Leu Ala Leu Arg His Ala Asp  
 85 90 95  
 Leu Pro Gly Pro Gln Arg His Val Phe Ser Asp Ala Trp Ser Asn Pro  
 100 105 110  
 Gln Leu Cys His Ile Ile Pro Leu Asp Arg Ala Phe Leu His Ala Ser  
 115 120 125  
 Asp Ala Asp His Thr Arg Trp Ala Arg Ser Ala Ser Ala Val Leu Gly  
 130 135 140  
 Ser Ala Gly Gly Ala Pro Ala Glu Gly Val Arg Glu His Ala Gly Arg  
 145 150 155 160  
 Val His Arg Glu Ala Ala Asp Arg Thr Gly Asp Ser Phe Asp Leu Met  
 165 170 175  
 Ala Asp Tyr Ser Arg Pro Val Ala Thr Glu Ala Ala Ala Glu Leu Leu  
 180 185 190  
 Gly Val Pro Ala Ala Gln Arg Glu Arg Phe Ala Ala Thr Cys Leu Ala  
 195 200 205  
 Leu Gly Val Ala Leu Asp Ala Ala Leu Cys Pro Gln Pro Leu Ala Val  
 210 215 220  
 Thr Arg Arg Leu Thr Glu Ala Val Glu Asp Val Arg Ala Leu Val Gly  
 225 230 235 240  
 Asp Leu Val Glu Ala Arg Arg Thr Gln Pro Gly Asp Asp Leu Leu Ser  
 245 250 255  
 Ala Val Leu His Ala Gly Ser Ser Ala Ala Ser Ala Gly Gln Asp Ala  
 260 265 270  
 Leu Ala Val Gly Val Leu Thr Ala Val Val Gly Val Glu Val Thr Ala  
 275 280 285  
 Gly Leu Ile Asn Asn Thr Leu Glu Ser Leu Leu Thr Arg Pro Val Gln  
 290 295 300  
 Trp Ala Arg Leu Gly Glu Asn Pro Glu Leu Ala Ala Gly Ala Val Glu  
 305 310 315 320

Glu Ala Leu Arg Phe Ala Pro Pro Val Arg Leu Glu Ser Arg Ile Ala  
 325 330 335  
 Ala Glu Asp Leu Thr Leu Gly Gly Gln Asp Leu Pro Ala Gly Ala Gln  
 340 345 350  
 Val Val Val His Val Gly Ala Ala Asn Arg Asp Pro Glu Ala Phe Leu  
 355 360 365  
 Ala Pro Asp His Phe Asp Leu Asp Arg Pro Ala Gly Gln Gly Gln Leu  
 370 375 380  
 Ser Leu Ser Gly Pro His Thr Ala Leu Phe Gly Ala Phe Ala Arg Leu  
 385 390 395 400  
 Gln Ala Glu Thr Ala Val Arg Thr Leu Arg Glu Arg Arg Pro Val Leu  
 405 410 415  
 Ala Pro Ala Gly Ala Val Leu Arg Arg Met Arg Ser Pro Val Leu Gly  
 420 425 430  
 Ala Val Leu Arg Phe Pro Leu Thr Thr Ser Ala  
 435 440

<210> 8  
 <211> 267  
 <212> PRT  
 <213> *Streptomyces galilaeus*  
 <400> 8  
 Val Asn Arg Ala Ala Arg Pro Thr Val Arg Gly Met Ser Ala Ile Ala  
 1 5 10 15  
 Glu Pro Thr Ala Pro Arg Gly Val Ile Val Thr Gly Gly Thr Gly  
 20 25 30  
 Ile Gly Arg Ala Thr Ala His Ala Phe Ala Asp Arg Gly Asp Arg Val  
 35 40 45  
 Leu Val Val Gly Arg Thr Ala Ala Thr Leu Ala Gly Thr Ala Glu Gly  
 50 55 60  
 His Pro Gly Ile Ser Val Leu Thr Ala Asp Leu Thr Asp Pro Asp Gly  
 65 70 75 80  
 Pro Arg Ala Ile Thr Asp Ala Ala Leu Asp Ala Leu Gly Arg Ile Asp  
 85 90 95  
 Val Leu Val Asn Asn Ala Ala Thr Gly Gly Phe Ala Gly Leu Ala Glu  
 100 105 110  
 Thr Glu Pro Glu Ala Ala Arg Glu Gln Phe Asp Ser Asn Leu Leu Ala  
 115 120 125  
 Pro Leu Leu Leu Thr Arg Gln Thr Leu Asp Ala Leu Ser Ala Asp Gly  
 130 135 140  
 Gly Gly Thr Val Leu Asn Ile Gly Ser Ala Gly Ala Leu Gly Arg Arg  
 145 150 155 160  
 Ala Trp Pro Gln Asn Gly Val Tyr Gly Ala Ala Lys Ala Gly Leu Asp  
 165 170 175  
 Phe Leu Thr Arg Thr Trp Ala Val Glu Leu Ala Pro Arg Gly Ile Arg  
 180 185 190

Val Leu Gly Leu Ala Pro Gly Val Ile Asp Thr Gly Ile Gly Glu Arg  
 195 200 205  
 Ser Gly Met Ser Arg Glu Ala Tyr Ala Gly Phe Leu Gly Gln Ile Ala  
 210 215 220  
 Ala Arg Val Pro Ala Gly Arg Val Gly Arg Pro Glu Asp Ile Ala Trp  
 225 230 235 240  
 Trp Ala Val Gln Leu Ala Asp Pro Arg Ala Ala Tyr Ala Thr Gly Ala  
 245 250 255  
 Val Leu Ala Val Asp Gly Gly Leu Ser Leu Thr  
 260 265

<210> 9  
 <211> 144  
 <212> PRT  
 <213> *Streptomyces galilaeus*

<400> 9  
 Met Thr Ala Gln Ala Pro Thr Ala Pro Ala Asp Val Tyr Ala Glu Val  
 1 5 10 15  
 Gln His Phe Tyr Ala Arg Gln Met Arg Tyr Leu Asp Ser Gly Glu Ala  
 20 25 30  
 Glu Thr Trp Ala Gly Thr Phe Thr Glu Asp Gly Ser Phe Ala Pro Pro  
 35 40 45  
 Ser Leu Pro Glu Pro Val Arg Gly Arg Pro Leu Leu Ala Glu Gly Ala  
 50 55 60  
 Arg Asn Ala Ala Ala Gly Leu Ala Ala Arg Glu Thr His Arg His  
 65 70 75 80  
 Trp Val Gly Met Leu Thr Val Thr Pro Ala Asp Asp Gly Ser Leu Thr  
 85 90 95  
 Ala Glu Ser Leu Val Ser Ile Val Ala Val Ala Gln Gly Gly Pro Ala  
 100 105 110  
 Arg Leu His Leu Val Cys Thr Cys Arg Asp Val Leu Val Arg Glu Gly  
 115 120 125  
 Gly Arg Leu Leu Val Arg Glu Arg Val Val Thr Arg Asp Asp Arg Pro  
 130 135 140

<210> 10  
 <211> 259  
 <212> PRT  
 <213> *Streptomyces galilaeus*

<400> 10  
 Val Arg Ile Ile Asp Leu Ser Ser Pro Val Asp Ala Ala Gly Phe Glu  
 1 5 10 15  
 Pro Asp Pro Val Val His Asp Val Leu Gly Pro Lys Glu Ala Ala Thr  
 20 25 30  
 His Met Ser Glu Glu Met Arg Glu His Phe Gly Ile Asp Phe Asp Pro  
 35 40 45

Ala Glu Leu Pro Glu Gly Glu Phe Leu Ser Leu Asp Arg Leu Gln Leu  
 50 55 60  
 Thr Thr His Thr Gly Thr His Val Asp Ala Pro Ser His Tyr Gly Thr  
 65 70 75 80  
 Arg Ala Ala Tyr Arg Asp Gly Pro Pro Arg His Ile Asp Glu Met Pro  
 85 90 95  
 Leu Asp Trp Phe Phe Arg Pro Ala Val Val Leu Asp Leu Ser Asp Gln  
 100 105 110  
 Gly Thr Gly Ala Val Gly Ala Asp Val Leu Arg Arg Glu Met Asp Arg  
 115 120 125  
 Ile Gly His Thr Pro Ser Pro Met Asp Ile Val Leu Leu Arg Thr Gly  
 130 135 140  
 Ala Asp Ala Trp Ala Gly Thr Pro Lys Tyr Phe Thr Asp Phe Thr Gly  
 145 150 155 160  
 Leu Asp Gly Ser Ala Val His Leu Leu Leu Asp Leu Gly Val Arg Val  
 165 170 175  
 Ile Gly Thr Asp Ala Phe Ser Leu Asp Ala Pro Phe Gly Asp Ile Ile  
 180 185 190  
 Thr Arg Tyr Arg Ala Thr Gly Asp Pro Ser Val Leu Trp Pro Ala His  
 195 200 205  
 Val Ile Gly Arg Asp Arg Glu Tyr Cys Gln Val Glu Arg Leu Ala Gly  
 210 215 220  
 Leu Asp Arg Leu Pro Ala Ala His Gly Phe Arg Val Ala Cys Phe Pro  
 225 230 235 240  
 Val Arg Ile Ala Gly Ala Gly Ala Gly Trp Thr Arg Ala Val Ala Leu  
 245 250 255  
 Val Asp Glu

<210> 11  
 <211> 238  
 <212> PRT  
 <213> *Streptomyces galilaeus*

<400> 11  
 Met Tyr Gly Arg Glu Leu Ala Asp Val Tyr Glu Ala Ile Tyr Arg Ser  
 1 5 10 15  
 Arg Gly Lys Asp Trp Gly Gln Glu Ala Ala Asp Val Ser Arg Ile Ile  
 20 25 30  
 Thr Glu Arg Arg Pro Gly Ala Gly Ser Leu Leu Asp Val Ala Cys Gly  
 35 40 45  
 Thr Gly Ala His Leu Ser Val Phe Ser Thr Leu Phe Glu Val Ala Glu  
 50 55 60  
 Gly Leu Glu Ile Ala Glu Pro Met Arg Arg Leu Ala Glu Gln Arg Leu  
 65 70 75 80  
 Pro Gly Thr Thr Val His Ala Gly Asp Met Arg Asp Phe Arg Leu Pro  
 85 90 95

Arg Thr Tyr Asp Ala Val Ser Cys Met Phe Cys Ala Ile Gly Tyr Leu  
 100 105 110  
 Glu Thr Leu Asp Asp Met Arg Ala Ala Val Arg Ser Met Ala Ala His  
 115 120 125  
 Leu Glu Pro Gly Gly Val Leu Val Val Glu Pro Trp Trp Phe Pro Glu  
 130 135 140  
 Asn Phe Ile Glu Gly Tyr Val Ala Gly Asp Leu Ala Arg Glu Glu His  
 145 150 155 160  
 Arg Thr Ile Ala Arg Ile Ser His Thr Thr Arg Lys Gly Arg Ala Thr  
 165 170 175  
 Arg Met Glu Val Arg Phe Thr Val Gly Asp Ala Ala Gly Ile Gln Gln  
 180 185 190  
 Phe Thr Glu Ile Asp Val Leu Thr Leu Phe Thr Arg Asp Glu Tyr Thr  
 195 200 205  
 Ala Ala Phe Thr Asp Ala Gly Cys Ser Val Glu Phe Leu Glu Asp Gly  
 210 215 220  
 Pro Thr Gly Arg Gly Leu Phe Val Gly Val Arg Glu Gln Arg  
 225 230 235

<210> 12  
 <211> 291  
 <212> PRT  
 <213> *Streptomyces galilaeus*

<400> 12  
 Met Lys Gly Ile Ile Leu Ala Gly Gly Ser Gly Thr Arg Leu His Pro  
 1 5 10 15  
 Ile Thr Val Ser Val Ser Lys Gln Leu Leu Pro Val Gly Asp Lys Pro  
 20 25 30  
 Met Ile Tyr Tyr Pro Leu Ser Val Leu Met Leu Ala Asp Ile Arg Glu  
 35 40 45  
 Ile Leu Leu Ile Cys Thr Glu Arg Asp Leu Glu Gln Phe Arg Arg Leu  
 50 55 60  
 Leu Gly Asp Gly Ser Gln Leu Gly Leu Arg Ile Asp Tyr Ala Val Gln  
 65 70 75 80  
 Asn Arg Pro Ala Gly Leu Ala Asp Ala Phe Val Ile Gly Ala Asp His  
 85 90 95  
 Val Gly Asp Asp Asp Val Ala Leu Val Leu Gly Asp Asn Ile Phe His  
 100 105 110  
 Gly His His Phe Tyr Asp Leu Leu Gln Ser Asn Val His Asp Val Gln  
 115 120 125  
 Gly Cys Val Leu Phe Gly Tyr Pro Val Glu Asp Pro Glu Arg Tyr Gly  
 130 135 140  
 Val Gly Glu Thr Asp Ala Ser Gly Gln Leu Val Ser Leu Glu Glu Lys  
 145 150 155 160  
 Pro Leu Arg Pro Arg Ser Asp Leu Ala Ile Thr Gly Leu Tyr Leu Tyr  
 165 170 175

Asp Asn Glu Val Val Asp Ile Ala Lys Asn Leu Arg Pro Ser Pro Arg  
 180 185 190  
 Gly Glu Leu Glu Ile Thr Asp Val Asn Arg Asn Tyr Leu Ala Arg Gly  
 195 200 205  
 Arg Ala Arg Leu Val Asp Leu Gly Arg Gly Phe Ala Trp Leu Asp Ala  
 210 215 220  
 Gly Thr Pro Glu Ser Leu Leu Gln Ala Thr Gln Tyr Val Arg Thr Leu  
 225 230 235 240  
 Glu Glu Arg Gln Gly Val Arg Ile Ala Cys Val Glu Glu Val Ala Leu  
 245 250 255  
 Arg Met Gly Phe Ile Asp Ala Asp Met Cys His Arg Leu Gly Glu Gln  
 260 265 270  
 Met Ser Gln Ser Gly Tyr Gly Arg Tyr Val Met Ala Val Ala Arg Glu  
 275 280 285  
 Phe Ser Gly  
 290

<210> 13  
 <211> 341  
 <212> PRT  
 <213> *Streptomyces galilaeus*

<400> 13  
 Met Thr Thr Leu Val Trp Asp Tyr Leu Gln Glu Tyr Glu Asn Glu Arg  
 1 5 10 15  
 Ala Asp Ile Leu Asp Ala Val Glu Thr Val Phe Ser Ser Gly Arg Leu  
 20 25 30  
 Val Leu Gly Asp Ser Val Arg Gly Phe Glu Glu Glu Phe Ala Ala Tyr  
 35 40 45  
 His Gly Ala Ala His Cys Val Gly Val Asp Asn Gly Thr Asn Ala Ile  
 50 55 60  
 Lys Leu Ala Leu Gln Ala Leu Gly Val Gly Pro Gly Asp Glu Val Val  
 65 70 75 80  
 Thr Val Ser Asn Thr Ala Ala Pro Thr Val Val Ala Ile Asp Ser Val  
 85 90 95  
 Gly Ala Thr Pro Val Phe Val Asp Val His Pro Asp Ser Tyr Leu Met  
 100 105 110  
 Asp Thr Glu Gln Val Glu Ala Ala Leu Thr Pro Arg Thr Arg Cys Leu  
 115 120 125  
 Leu Pro Val His Leu Tyr Gly Gln Cys Val Asp Leu Ala Pro Leu Glu  
 130 135 140  
 Arg Leu Ala Ala Glu His Asp Leu Phe Leu Val Glu Asp Cys Ala Gln  
 145 150 155 160  
 Ala His Gly Ala Arg Arg Ala Gly Arg Leu Ala Gly Thr Thr Gly Asp  
 165 170 175  
 Ala Ala Ala Phe Ser Phe Tyr Pro Thr Lys Val Leu Gly Ala Tyr Gly  
 180 185 190

ggccggaccgc gcctgaagac cggtgaggga ccggtaggcc cgttcggggc ggtcgcccc 1800  
 gtctctggga taggcgcaca gcagtcggc gtccacgcg cgggtcacga gcccgtcgcc 1860  
 gatgtccggcc ggcagccgg gtcccgctc ggcacatcg cccggcagat cgggctgccc 1920  
 gtcgagcagc gggccacga gggcgatctg ctccccgccc agcgtggta cccggctctc 1980  
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 cagccggggg agcccggtc cagggggccgt acggctcatc gagacgcagg gcccggacc 2100  
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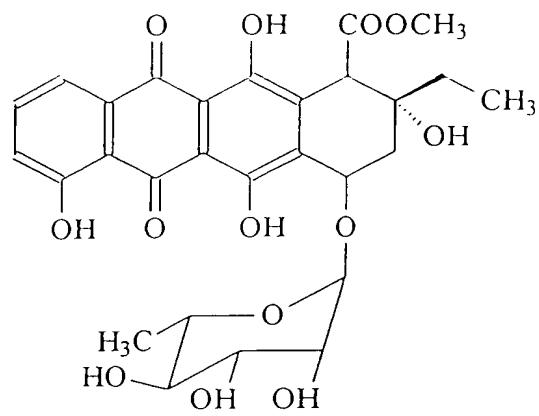
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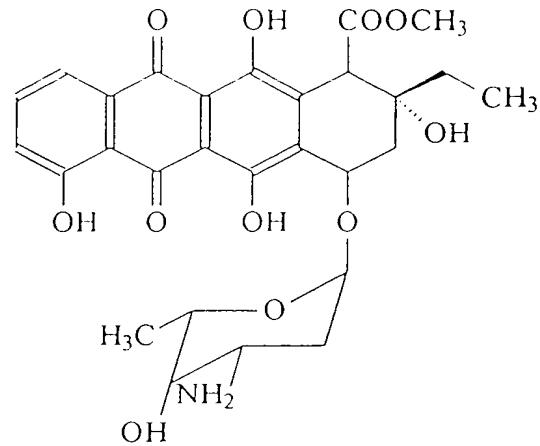
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Figure 4



1, L-rhamnosyl- $\epsilon$ -rhodomycinone



2, L-daunosaminyl- $\epsilon$ -rhodomycinone

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